EVALUATION OF ANTI-HLA-DR MONOCLONAL ANTIBODY THERAPY IN SPONTANEOUS CANINE LYMPHOMA

Rhona Stein,[1]* Cheryl Balkman,[2]* Susan Chen,[1] Kenneth Rassnick,[2] Margaret McEntee,[2] Rodney Page,[2] David M. Goldenberg[1]

[1]Garden State Cancer Center, Center for Molecular Medicine and Immunology, Belleville NJ, U.S. [2]Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, U.S.

*These authors contributed equally.

Running title: Anti-HLA-DR Therapy in Canine Lymphoma

Keywords: Monoclonal antibody, HLA-DR, L243, IMMU-114, dog, lymphoma

Grant Support: This work was supported in part by grant P01-CA103985 from the National Cancer Institute, N.I.H., and the Canine Health Foundation, American Kennel Club.

Financial conflicts: Dr. Goldenberg is an officer and holds stock in Immunomedics, Inc. The other authors declare no financial conflicts.

Requests for reprints: Rhona Stein; Garden State Cancer Center, Center for Molecular Medicine and Immunology, 520 Belleville Ave, Belleville, NJ 07109; Tel.: (973) 844-7012; fax: (973) 844-7020; E-mail: rstein@gscancer.org.

Statement of Translational Relevance: This paper presents a preliminary clinical evaluation of anti-HLA-DR monoclonal antibody therapy for treatment of canine B-cell lymphoma. *In vitro* studies show that murine L243 and its humanized IgG4 construct, IMMU-114, bind to normal and malignant canine lymphocytes and subsequently induce biological activity. *In vivo* studies indicate that the murine and humanized mAbs can be administered safely to dogs with lymphoma and bind to the malignant cells in nodal tissue. Preliminary evidence of disease stabilization was observed in dogs with advanced-stage lymphoma following anti-HLA-DR immunotherapy. These findings provide a strong rationale for the use of both normal dogs and dogs with lymphoma in preclinical safety and efficacy evaluations of IMMU-114 or other anti-HLA-DR mAbs for both veterinary applications and for preclinical testing in preparation for human trials.

ABSTRACT

Purpose: Expression of HLA-DR on hematological malignancies generated considerable interest in its development as a target for antibody-based therapy. Here we describe the use of anti-HLA-DR monoclonal antibodies (mAbs), L243 and IMMU-114 (hL243γ4P), a humanized IgG4 mAb engineered to eliminate adverse reactions associated with complement-activation, for antibody therapy in dogs with lymphoma.

Experimental Design: Normal and malignant canine B-cell binding, induction of apoptosis, antibody-dependent cellular-cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and direct cytotoxicity of L243 and IMMU-114 were measured *in vitro*. Safety and pharmacokinetic data on L243 and IMMU-114 administration were collected in normal dogs, followed by a preliminary trial of L243 in dogs with advanced lymphoma or unresectable plasmacytoma.

Results: L243 and IMMU-114 bind to normal canine lymphocytes and canine lymphoma cells. *In-vitro,* murine L243 and IMMU-114 binding yielded reduction in viable cell counts and induction of apoptosis in canine lymphoma cells. When incubated with canine serum or peripheral blood mononuclear cells, L243, but not IMMU-114, induced CDC and ADCC, respectively. *In-vivo,* both anti-HLA-DR mAbs can be administered safely to dogs and bind to the malignant cells. Evidence of clinical activity (hematopoietic toxicity and tumor response) was observed in dogs with advanced-stage lymphoma following L243 immunotherapy.

Conclusions: This study demonstrates the value of canine lymphoma for safety and efficacy evaluations of IMMU-114 or other anti-HLA-DR mAbs for both veterinary applications and for preclinical testing in preparation for human trials.

INTRODUCTION

Rituximab anti-CD20 IgG therapy is credited with revitalizing antibody therapies with its ability to effectively treat follicular lymphoma without the extensive side effects associated with more traditional chemotherapy regimens. Since rituximab's approval by the FDA in 1997, the mortality rate from NHL has declined by 2.8% per year (1), and the use of this agent has been expanded to a variety of diseases (2-10). While rituximab has been a remarkable success in follicular non-Hodgkin lymphoma (NHL), for which it was first approved, only half of the patients had an objective response, with at most 10% having a complete response (11). Rituximab was less effective in the more aggressive types of NHL, such as diffuse large B-cell lymphoma (DLBCL), but when it was combined with combination chemotherapy, improved and durable objective responses compared to the separate therapies were found, making R-CHOP a standard protocol for the treatment of DLBCL (7, 12-14). The success of rituximab stimulated the evaluation of a number of other antibodies and antibody conjugates, and while a number of these have shown promising activity, to-date only one other unconjugated antibody therapy, alemtuzumab anti-CD52 for chronic lymphocytic leukemia (CLL), has been approved for use in hematologic malignancies (15).

Novel immunotherapeutic approaches, such as infusion of monoclonal antibodies (mAbs) to improve the management of lymphoma, are traditionally examined in murine models for efficacy and in Cynomolgus monkeys for safety and pharmacodynamics/pharmacokinetics, but could be more carefully evaluated prior to human studies to identify and better anticipate the impact of such interventions in humans if other tumor models were available. Spontaneously-arising neoplasms in companion animals have been proposed as a useful system for examining numerous hypotheses relevant to human cancer control, and have recently been acknowledged by the National Cancer Institute as valuable study models (16-18). Canine lymphoma is

particularly valuable, because it is common (incidence 25-40/100,000), similar pathologically to human high-grade, B-cell NHL, and is initially controllable with chemotherapy followed by subsequent resistance that limits long-term control (median remission and survival times are 6-9 months and 10-14 months, respectively) (19). Recent investigations have confirmed that canine lymphoma is genetically similar to the human disease (20, 21), further justifying the model for evaluating novel, targeted therapeutic strategies for the benefit of both species.

The human leukocyte antigen–DR (HLA-DR) is one of three isotypes of the major histocompatibilty complex (MHC) class II antigens. HLA-DR is highly expressed on a variety of hematologic malignancies and has been actively pursued for antibody-based lymphoma therapy (22-24). Anti-HLA-DR mAbs are markedly more potent than other naked mAbs of current clinical interest in *in vitro* and *in vivo* experiments in lymphomas, leukemias, and multiple myeloma.[3] HLA-DR is also expressed on a subset of normal immune cells, including B cells, monocytes/macrophages, Langerhans cells, dendritic cells, and activated T cells (25). Thus, it is perhaps not surprising that infusional toxicities, likely related to complement activation, have been problematic clinically with the administration of anti-HLA-DR antibody (26).

To avoid complications associated with complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), we recombinantly-engineered a humanized IgG4 construct of the murine anti-HLA-mAb, L243, referred to as hL243γ4P (IMMU-114) (24). The IgG4 isotype was prepared because human Fcγ receptors are known to have low affinity for the human IgG4 isotope (27). A point mutation, Ser241Pro, was introduced into the hinge region of the γ4 sequence in order to avoid formation of half-molecules when the antibody is expressed and produced in mammalian cell cultures, thus explaining the designation, γ4P. Replacing the Fc region of a humanized IgG1 anti-HLA-DR mAb with the IgG4 isotype abrogated the effector cell

functions of the antibody (ADCC and CDC), while the antigen-binding properties, anti-proliferative capacity (*in vitro* and *in vivo*), and the ability to induce apoptosis concurrent with activation of the AKT survival pathway and other signaling pathway effects, were retained (24). Thus, IMMU-114 is indistinguishable from the parental murine mAb and a humanized IgG1 anti-HLA-DR mAb in assays dependent upon antigen recognition, and the abrogation of ADCC and CDC may make this antibody an attractive clinical agent so long as the signaling effects observed *in vitro* and in human lymphoma xenografts models[3] (24) are retained in patients.

This work describes the use of anti-HLA-DR mAbs in dogs with lymphoma to confirm the suitability of this model to address therapeutically relevant endpoints prior to human clinical investigation. The *in-vitro* studies show that murine L243 and its humanized IgG4 construct, IMMU-114, bind to normal and malignant lymphocytes and subsequently induce biological activity. *In-vivo* studies indicate that the murine and humanized mAbs can be administered safely to dogs with lymphoma and bind to the malignant cells in nodal tissue. Preliminary evidence of disease stabilization was observed in dogs with advanced-stage lymphoma following anti-HLA-DR immunotherapy. These observations enable further studies in dogs to refine dosing schemes as well as characterize safety and efficacy endpoints of anti-HLA-DR mAbs for both veterinary applications and in preparation for human clinical trials.

MATERIALS AND METHODS

Antibodies

The following mAbs were used for phenotyping: anti-CD3-FITC, anti-CD4-FITC, anti-CD8-PE, and B-cell-PE, purchased from Serotec Ltd (Raleigh, NC), unlabeled anti-human CD22 (LL2) and anti-human CD74 (LL1), supplied by Immunomedics, Inc. (Morris Plains, NJ),

unlabeled anti-human-CD3, -CD20, and -CD45 (Leu 4, Leu-16, and H-Le-1, respectively), purchased from BD Biosciences (San Jose, CA), and anti-CD20 mAbs, 2B8 and 1F5, purified from culture fluids of hybridoma cells obtained from the American Type Culture Collection (ATCC, Manassas, VA). Murine mAbs Ag8 (P3x63 Ag8, ATCC) and MN-14 (anti-carcinoembryonic antigen [CEA, CEACAM5 or CD66e]) were used as isotype controls. L243 and humanized mAbs, IMMU-114 (hL243γ4P) (24), milatuzumab (hLL1, anti-CD74 mAb) (28), veltuzumab (hA20, anti-CD20) (29), epratuzumab (hLL2, anti-CD22) (30), and labetuzumab (hMN-14, anti-CEA) (31), were provided by Immunomedics, Inc.

Flow cytometry

Peripheral blood lymphocyte subsets were determined using flow cytometry. The different leukocyte populations were identified by their distinctive position on forward and side scatter plots. The lymphocyte population was gated and 10,000 events were acquired for each antibody. All flow cytometry experiments were performed and analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA). The data were analyzed with CellQuest software. Immunostaining was performed according to the manufacturer's directions. Briefly, a 100-µl aliquot of whole blood in EDTA was incubated with either antibody or isotype control antibody for 15 min at room temperature. Red blood cells were lysed with 2 ml of FACS lysing solution and incubated for 5 min. The cells were washed in phosphate-buffered saline (PBS), pH 7.4. The cell pellet was resuspended in PBS containing 20 mM glucose and 1% bovine serum albumin and immediately assayed by flow cytometry.

Determination of HLA-DR and CD20 antigen expression on normal and neoplastic cells was performed by indirect immunofluorescence assays using FITC-goat anti–mouse IgG (GAM, Invitrogen, Carlsbad, CA), as described previously (24).

In vitro cytotoxicity and apoptosis assays

Apoptosis was evaluated by flow cytometry as described previously (29). Briefly, cells were incubated with mAbs for 48 h with or without a second antibody for cross-linking, followed by DNA staining with propidium iodide. Samples were analyzed by flow cytometry using a FACSCalibur. Percentage of apoptotic cells was defined as the percentage of cells with DNA staining before G1/G0 peak (hypodiploid).

Standard ^{51}Cr release assays measured ADCC and CDC, essentially as described (32). Briefly, for CDC a 1/8 final dilution of canine serum was used as the source of complement, followed by a 3-h incubation. Cells treated with 0.25% Triton X-100 were included as 100% lysis control, and cells treated with complement alone as 0% lysis. For ADCC, effector:target cell ratios of approximately 50:1 were used, and incubations were for 4 h. All assays were performed in triplicate.

In vivo studies

Dog studies were approved by Cornell University's Institutional Animal Care and Use Committee. The sterile antibodies were diluted in a total volume of 90 ml and 250 ml of 0.9% NaCl for administration to normal dogs and dogs with lymphoma, respectively. All dogs were pre-medicated with 4 mg/kg diphenhydramine intramuscularly 20 min prior to antibody infusion. The dogs were monitored continuously during the infusion, and vital signs and body temperature were recorded every 30 min. If adverse events (vomiting, erythema, pruritus, weakness, tachycardia) were observed, the infusion was stopped for at least 15 min and restarted at half the initial infusion rate. The normal dogs' rectal temperature was taken twice daily for 7 days following each antibody infusion. Adverse events were graded according to the veterinary co-operative group – common terminology criteria (33).

Normal dogs. Intact female beagle dogs were used to assess systemic toxicity with L243 (2 dogs) and IMMU-114 (2 dogs) antibody administration. The dogs were deemed healthy based on physical examination, complete blood cell count, biochemical profile, and urinalysis. L243 was administered at 1.5 mg/kg of body weight over a planned 90-min interval to the first dog. This dose was extrapolated from previous dose ranging studies in mice. The antibody infusion was repeated on day-7 in this dog. The dose of L243 was increased to 3 mg/kg for the second dog, since there was minimal toxicity noted in dog-1. The second dose was repeated on day-2 rather than day-7, to determine if increased toxicity would be detected with a shorter interval between treatments. IMMU-114 was administered at 3 mg/kg infused over a 90-min period to 2 dogs. One of the dogs was infused a second time 2 weeks later at 1.3 mg/kg.

Blood was collected into ethylenediamine tetraacetic acid (EDTA) tubes for complete blood cell counts and peripheral blood lymphocyte phenotyping at 4, 24, 48, and 72 h and at 7, 14, 21 days after the first infusion. Biochemical profiles and urine were analyzed at 7, 14, and 21 days after the first infusion. Dogs were humanely euthanized by intravenous pentobarbital sodium injection 30 days after the first infusion. Necropsies were performed post mortem and tissue samples were collected in formalin for histologic review by a board certified pathologist.

Dogs with Lymphoma. Dogs were enrolled in this study if they had histologic or cytologic confirmation of lymphoma or plasma cell neoplasia and had previously failed or were refractory to conventional cytotoxic chemotherapy or if the owner had declined other therapy. Chemotherapy was not administered concurrently or less than 3 weeks prior to treatment with HLA-DR mAb. Written, informed consent was obtained from all owners prior to treatment. Pretreatment evaluation for all tumor-bearing dogs included physical examination, complete blood cell count, biochemical profile, and urinalysis. Dogs were excluded if there was evidence

of ≥ grade 2 toxicity on screening studies. Lymph nodes or tumors were measured in 3 dimensions and tumor volume was calculated as the product of length, width, height, π/6. Dogs received 1 to 4 treatments administered at 2-week intervals at a dosage of 3 mg/kg intravenously. Based on the normal dog studies above, the starting protocol for infusion of L243 was planned over a 4-h period. Due to delays caused by infusion reaction in some of the dogs, the infusion was slowed to 3 mg/kg over 12 h. Adverse infusion events were monitored continuously in an intensive care setting during the infusion. Complete blood cell count, chemistry profile, urinalysis and tumor measurements were evaluated weekly.

Enzyme-linked immunoabsorbent assay (ELISA)

L243 and IMMU-114 serum levels were measured by ELISA. Two ml of whole blood were collected pretreatment, at the end of the antibody infusion, 1 h after the end of the infusion and at 24 h. The samples were allowed to clot at room temperature for 30 min and the serum was separated and frozen at -80^0 C prior to analysis. The ELISA assays were performed in 96-well PVC microtiter plates. Plates were coated overnight with goat anti-mouse IgG F(ab')$_2$ fragment specific antibody at 10 μg/ml in PBS, 0.02% NaN_3 (Jackson Immunoresearch, West Grove, PA), then blocked with 1% BSA/PBS, 0.02% NaN_3 for 1 h at room temperature. Triplicate serum dilutions (in 1% BSA/PBS, 0.02% NaN_3 at 1/3, 1/10, 1/30, and 1/100) were incubated for 1 h in the coated wells. A standard curve of L243 or IMMU-114 was run in the same plate. After washing with PBS, 0.05% Tween, peroxidase conjugated goat anti-mouse (or anti-human) IgG, Fcγ specific antibody (1:3000 dilution in 1% BSA/PBS, Jackson Immunoresearch) was added and the plate was incubated for an additional 1 h at room temperature in the dark. The plates were washed, developed with o-phenylenediamine dihydrochloride substrate solution and read at 490 nm, after stopping the reaction by adding 1.5 N H_2SO_4.

Statistics

P-values were calculated using the Student's *t* test. Two-sided tests were used throughout. Values less than 0.05 are considered statistically significant. For ADCC and CDC assays, *P* values were calculated versus the no-antibody control.

RESULTS

Selection of HLA-DR as target antigen

Cross-reactivity of a panel of anti-human B-cell mAbs with dog lymphocytes was evaluated using peripheral blood from a healthy dog. A human blood sample was tested at the same time as a control. Single color indirect flow cytometry analysis was performed. Reactivity of the mAb panel with the human lymphocytes was within the expected range. MAbs against human CD20 (1F5) and HLA-DR (L243) reacted with the dog lymphocytes. Anti-human CD22 (LL2), CD74 (LL1), and mAbs recognizing human CD3 (Leu 4), CD20 (Leu-16), and CD45 (H-Le-1) did not cross-react with dog lymphocytes. Based on these initial results, tumor aspirates obtained through a large gauge needle from dogs with lymphoma were tested for binding to anti-HLA-DR and anti-CD20 murine mAbs. Anti-HLA-DR (L243) was positive in 32/35 samples (greater than 5 units above the isotype control) and strongly positive (greater than 10 units above the negative control) in 30/35 samples. In contrast, anti-CD20 (2B8 used in these studies) was positive in 5/21, including 3 strongly positive. Reactivity of L243 was confirmed on the peripheral blood of several of these dogs. Thus, L243 was selected for further evaluation of potential therapeutic effects in canine lymphoma.

In vitro effects of L243 on proliferation and apoptosis

Lymph node aspirates from four dogs with lymphoma were incubated with mAb L243 *in vitro* to determine the effects of the mAb on proliferation and apoptosis. All four specimens were positive for L243 binding (**Figure 1A**). Induction of apoptosis by L243 was evaluated by flow cytometry assays measuring hypodiploid DNA. Cells were cultured with the mAbs for 48 h with or without a second mAb for cross-linking, followed by DNA staining with propidium iodide. Cells were analyzed by flow cytometry, and positive fluorescence below the G0/G1 region represents DNA fragmentation and is a measure of apoptosis. As shown in **Figure 1B**, L243 caused specific induction of apoptosis in the presence of goat anti-mouse IgG second antibody ($P<0.05$ vs. crosslinked isotype control) in all four specimens. Viable cell counts were measured after 2-day incubations of the tumor aspirates with L243 plus goat anti-mouse IgG second antibody. Decreases in the viable tumor cell population of 43% ($P=0.0088$) and 23% ($P=0.097$) were obtained in specimens 160812 and 160965, respectively, *vs.* Ag8 plus goat anti-mouse IgG second antibody (**Figure 1C**). Specimens 160540 and 150836 were not tested by this assay. ADCC and CDC assays were performed on one tumor aspirate, from dog 171205, using PBMCs or serum isolated from that animal as sources of effector cells and complement, respectively. Statistically significant lysis was observed with L243 but not an isotype control (MN-14) in both assays. For CDC, lysis was 38.1%±0.9% ($P=0.0004$) and 1.1±2.2% ($P=1.0000$) for L243 and isotype control, respectively. For ADCC, lysis was 26.6±15.9% ($P=0.0319$) and -6.9±18.36% ($P=0.4544$) for L243 and isotype control, respectively. Thus, crosslinked L243 yields a specific therapeutic effect on canine lymphoma aspirates, leading to a reduction in viable cell count and induction of apoptosis, as measured by DNA fragmentation. When incubated with dog serum or PBMCs, L243 induces CDC and ADCC.

We also demonstrated that IMMU-114 (humanized, engineered L243) binds to canine lymphoma cells **(Table 1)**. In addition, IMMU-114 induces apoptosis in the canine lymphoma cells when crosslinked with an anti-human IgG second antibody (**Figure 2A**). Evaluation of the

ability of IMMU-114 to induce CDC and ADCC was performed on one canine lymphoma aspirate (171205). As shown in **Figures 2B and C**, murine L243 but not IMMU-114 yielded specific cell lysis of the dog lymphoma cells, confirming the lack of CDC and ADCC effector functions of IMMU-114.

L243 administration in vivo

Safety data on L243 infusion was collected in two normal dogs, followed by a trial in 6 dogs with relapsed lymphoma, and 1 dog with an unresectable plasmacytoma.

Normal dogs. Dog 1 received 2 infusions of 1.5 mg/kg, 7 days apart. An infusional reaction occurred during the first antibody administration that included grade I nausea/vomiting and grade I fever. Decreasing the infusion rate by 50% (from an initial rate of 0.2 mg/ml/min) eliminated the adverse reactions. There were no adverse events during the second infusion. Dog 2 received 2 infusions of 3.0 mg/kg, 48 hours apart (0.25 mg/ml/min). There were no adverse reactions during either infusion. There were no significant changes in the post-infusion biochemical profiles or urinalysis in either dog. Mature neutrophils were transiently elevated in Dog 2 (13.3 x 10^3/μl; normal range 3.4 – 9.7 x 10^3/μl) 24 h after the first infusion and normalized within 24 h. Both dogs had a marked transient increase in band neutrophils. Dog 1 had 1000/μl band neutrophils 4 h after the second infusion (normal range 0-100/μl); Dog 2 had 1300/μl band neutrophils 24 h after the first infusion. Both dogs had normal band neutrophil counts 24 h later. Lymphopenia (800/μl - dog 1, 500/ μl – dog 2: normal range 1000 – 4000/ μl) was noted 4-24 h following the first infusion in both dogs and following the second infusion in dog 2. Lymphocytes returned to normal within approximately 1 week following infusion. Peripheral blood lymphocyte subset phenotyping indicated a decrease in both B and T cell lymphocytes (**Figure 3**). Such rapid changes in neutrophils and lymphocytes represent a non-specific component to immunogens in dogs. Resolution of the neutrophilia occurred within one day and lymphocyte

populations recovered over a 7-day period. Complete necropsy examination of Dogs 1 and 2 did not reveal any gross or histologic abnormalities.

Tumor-bearing dogs.

Patient characteristics. Seven dogs with lymphoma/plasmacytoma were treated with L243. The median age of the patients was 10.8 years (range 8.4 – 11.9 years). The median body weight was 35 kg (range 12.6 – 51.2 kg). There were 4 male dogs (2 intact, 2 castrated), and 3 female dogs (all spayed). Four of the dogs had B-cell lymphoma, 2 had T-cell lymphoma, and 1 had an unresectable plasmacytoma. All dogs were staged according to WHO guidelines for canine lymphoma: 3 were stage V, 2 were stage III, and one remained incompletely staged. Four of the six lymphoma patients had failed initial conventional and rescue chemotherapy treatments; the remaining two lymphoma patients had received prednisone as their only therapy prior to presentation and their owners' had declined standard chemotherapy. All previous chemotherapeutic agents were discontinued 2-4 weeks prior to L243 therapy.

Toxicity. Infusional side-effects were common with 6/7 patients, experiencing grade 1 nausea or vomiting and 5/7 experiencing grade 1 fever. Slowing the infusion rate abrogated the adverse reactions. Two dogs received dexamethasone at 0.5 – 2 mg/kg i.v. due to vomiting and elevated temperature. No dog had treatment discontinued due to adverse events. Hematologic toxicity was noted in 3/7 patients. One dog had grade 1 neutropenia and grade 1 thrombocytopenia two weeks after the first infusion. This dog received a total of 3 treatments and did not exhibit any additional hematologic abnormalities. In two dogs, grade 3 neutropenia and grade 4 thrombocytopenia were observed one week after the second infusion. Both of these dogs were heavily pretreated with chemotherapy prior to antibody infusion. Bone marrow aspirates indicated a non-specific granulocytic and megakaryocytic hypoplasia. One dog was euthanized due to hemorrhage from multiple ulcerated cutaneous lymphoma lesions. The

second dog's cytopenias resolved uneventfully by the fourth week post infusion. One dog died suddenly at home approximately 5 days after L243 therapy due to rapidly progressing, resistant lymphoma. A necropsy was not performed.

Response to therapy. Two dogs with advanced, multicentric B-cell lymphoma had a transient response to L243 therapy. One dog had stable disease with complete resolution of circulating lymphoblasts for 5 weeks following the second infusion, with improvement in attitude and appetite. This dog received a total of three treatments. His disease progressed 8 weeks after his first L243 treatment. The second dog had a 50% reduction in the size of peripheral lymph nodes observed by physical examination and measurement of peripheral lymph node volume one week after the first treatment. The partial response lasted 8 weeks before progressive disease was noted. This dog received a total of 4 treatments without evidence of any toxicity. Both dogs received a brief course (1-2 weeks) of corticosteroid prior to L243 therapy. In each instance, the dogs had progressive disease on corticosteroids prior to L243 infusion and all corticosteroid therapy was discontinued before treatment.

A comparison of cells aspirated from a lymph node prior to L243 with cells obtained one week after the first L243 infusion was performed in order to assess *in vivo* targeting of the L243 mAb. The histograms shown in **Figure 4A** represent baseline and one-week post infusion aspirated cells, to which no first or second antibodies were added *in vitro*. The profiles of the baseline cells and week-1 cells overlap. The cells in **Figure 4B** were incubated *in vitro* with FITC-labeled GAM, to detect cells that were labeled with L243 *in vivo*. Cells obtained from the same lymph node 1 week after treatment with L243 are shifted to the right of the baseline cells demonstrating the binding of murine IgG to the cell surface. The cells in **Figure 4C** were incubated *in vitro* with L243 and FITC-GAM to determine whether the cells were saturated with mAb L243. Aspirated cells taken 1 week after treatment with L243 coincide with the baseline

cells because the *in vivo* and *in vitro* binding of L243 IgG to the cell surface are indistinguishable after saturating doses of L243. Both groups exhibited higher mean fluorescence compared to that of the FITC-GAM labeled cells in **Figure 4B**, indicating that the *in vivo* L243 dose administered did not saturate all malignant cells in the node. Data obtained from cells aspirated 2 weeks after infusion continue to demonstrate L243 binding to malignant lymphocytes. An alternate explanation is that some of the bound L243 was internalized or processed, and the antigen remains on or returns to the cell surface, able to bind additional antibody. Cells in **Figure 4D** were incubated *in vitro* with Ag8 (isotype matched, nonspecific mAb) and FITC-GAM. Aspirated cells taken 1 week after infusion with L243 are shifted to the right of the baseline cells, similar to the profile observed in **Figure 4B**, again demonstrating the binding of murine IgG to the cell surface. Only the cells labeled *in vivo* with L243 bind to the FITC-GAM, because Ag8 does not bind to the cells. This assay demonstrated that L243 targeted the tumor cells *in vivo*.

The L243 antibody was measured by ELISA in the serum of the last treated dog (152616). Samples were collected prior to the antibody infusion, at the end of the infusion, 1 h post infusion and at 24 h at each of the 4 treatments (**Figure 5**). The serum level of L243 detected after the second infusion was markedly higher than after the first infusion. This suggests that the antigen pool present on cell surfaces was either blocked or eliminated by the first infusion. Infusions 3 and 4 yielded progressively lower serum concentrations of L243, this is likely due to an anti-antibody response causing rapid clearance of the infused murine L243 antibody. Because the presence of anti-mouse IgG was not measured, reappearance of an antigen sink cannot be ruled out.

IMMU-114 administration in vivo

Once IMMU-114, the humanized reengineered IgG4 form of murine L243, became available, it was administered to 2 normal beagles at 3 mg/kg over 90 min. There was no

infusion reaction noted in either dog during the infusion. One of the dogs was infused a second time 2 weeks later (at 1.3 mg/kg). A mild infusion reaction that included head shaking, mild fever and vomiting occurred following the second infusion. The severity of the reaction was lessened by slowing the rate of the infusion. This may suggest the development of anti-human IMMU-114 antibody. CBCs and biochemical panels were conducted with no significant changes noted over a 2-week period, with the exception of a transient lymphopenia as also observed with L243 infusion. Pharmacokinetic (PK) data obtained at the end of infusion, and 1, 4, 24, 48, 72 h, 1 week, and 2 weeks post-infusion indicated a rapid clearance within the first few hours, with about 50% of the IMMU-114 antibody cleared within 2 h, and with the remaining antibody clearing with a half-life of ~2 days (**Figure 5**).

DISCUSSION

Naturally-occurring lymphoma in dogs is extremely common and has been validated as a useful model of high-grade, B cell, non-Hodgkin's lymphoma in humans. Conventional chemotherapeutic management of lymphoma in dogs, as in humans, is limited with 5-20% 2-year survival rates following CHOP-based chemotherapeutic protocols. The availability of canine lymphoma patients, the ability to investigate novel strategies with repeated sampling of normal and tumor tissue or fluid, as well as the design of rigorous clinical trials to determine relevant therapeutic endpoints, are recognized advantages of this model as a bridge from preclinical investigations to humans. Although anti-CD20 antibodies have contributed to improved outcomes in some forms of lymphoma in humans, the commercially available human anti-CD20 antibodies do not bind sufficiently with canine B-cell lymphomas to permit further investigations of this strategy. However, substantial opportunities exist to expand the investigation of other antibody-based immunologic therapeutics.

Lymphoma is an increasingly common form of cancer with a wide range of immunologic and genetic subcategories with equally diverse prognoses. Aggressive forms of non-Hodgkin's lymphoma are currently controlled with chemotherapy with or without antibody infusions with only a moderate degree of success. Novel immunotherapeutic approaches, such as infusion of anti-B-cell mAbs to improve the management of lymphoma, are traditionally examined in murine models but should be more carefully evaluated prior to human study to identify and better anticipate the impact of such interventions. The studies reported in this paper were performed to develop a solid rationale and expedited process for human clinical evaluation of IMMU-114, a humanized IgG4 engineered mAb based on the murine anti-HLA-DR mAb, L243. Studies in this canine model are important to the translation of IMMU-114 to clinical studies in humans, particularly given the prior clinical experience with another anti-HLA-DR antibody (Hu1D10; apolizumab), where moderate to severe side effects, primarily related to robust immune effector activity (e.g., mainly CDC) limited its dosing (26). Apolizumab is not being actively pursued clinically. In order to expedite the scientific and practical decisions about progression of new immunotherapeutic strategies into humans with B-cell malignancies, prudent use of the canine lymphoma model to address both safety and efficacy represents a truly comparative approach to cancer investigation.

The effects of anti-HLA-DR antibodies on malignant cells have been studied extensively. The most widely recognized function of class II major histocompatibilty complex (MHC) molecules is the recognition of foreign antigen fragments and presentation to CD4 T lymphocytes. In addition, signals delivered via HLA-DR molecules contribute to the functioning of the immune system by up-regulating the activity of adhesion molecules, inducing T-cell antigen counter receptors, and initiating the synthesis of cytokines. Stimulation of HLA molecules by antibodies has been shown to affect growth, differentiation, and immunoglobulin secretion by B lymphocytes, as well as production of cytokines, modulation of expression of growth factor receptors, cell adhesion, and co-stimulatory molecules by B cells and monocytes (34). HLA molecules have also been shown to serve as

receptors that activate various cell death pathways, including caspase-dependent and caspase-independent alternative pathways of apoptosis (34-37). Functions reported to be affected by incubation of cells with L243 have included signal transduction, growth inhibition, Fas-mediated apoptosis, interactions with actin microfilaments, TNF-α and TNF-β gene expression, cell adhesion, ADCC, and others (34-46). Enhanced cell kill over rituximab alone is demonstrated when the IMMU-114, is combined with rituximab *in vitro* (24). Recent studies have shown that antigen expression is not sufficient for cytotoxicity, but that antibody-induced activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) stress signaling pathways are also required.[3]

The results reported here show that the anti-HLA-DR antibodies, L243 and IMMU-114, are able to induce cell death of canine lymphoma cells *in vitro* and can be given safely to dogs with lymphoma that are not heavily pretreated with chemotherapy. From this study, we were able to obtain valuable information regarding the dose and infusion rate for canine patients diagnosed with B-cell lymphomas. The primary reaction following initiation of the infusion was mild and was characterized by a grade 1 fever and grade 1 nausea/vomiting. Myelosuppression was only noted in canine patients that were heavily pretreated with other chemotherapeutic agents. No other severe acute reactions were observed.

Two dogs with T-cell lymphoma were treated. Our preliminary work demonstrated that the T-cell form of lymphoma did not bind L243 significantly. We chose to enroll these dogs to identify whether the infusion reaction may be non-specific in an L243-negative tumor. Neither dog expressed the L243 antigen on the tumor cells. Both of these dogs experienced similar infusion reactions to those dogs with L243+ B-cell tumors.

All dogs had tumor measurements and were evaluated for response, although this study was not designed to evaluate true response probability. The two dogs with B-cell lymphoma that

had received prednisone as their only prior therapy experienced measurable responses to L243. One experienced a minor, but measurable, response with significant improvement of advanced symptoms, while the second had a partial response lasting 8 weeks. Five dogs did not demonstrate an obvious tumor response. Dogs in this group were L243-negative (T-cell lymphoma) or had end-stage disease at the time of treatment. Future studies focusing on enrollment of dogs with early B-cell lymphoma may provide further support for efficacy of this antibody.

The initial studies reported here raise a number of questions for future studies. Such issues include the immunogenicity of the murine L243 and humanized IMMU-114 mAbs and whether a caninized form is more suitable for future canine studies, the optimal dose and schedule, and whether combining IMMU-114 with standard cytotoxic drugs, as has been the experience with most anti-cancer antibodies in current clinical use, may show more potent anti-lymphoma results than single-modality regimens. Nevertheless, these findings provide a strong rationale for the use of both normal dogs and dogs with lymphoma in preclinical safety and efficacy evaluations of IMMU-114 or other anti-HLA-DR mAbs for both veterinary applications and for preclinical testing in preparation for human trials.

FOOTNOTE

[3]Stein R, Gupta P, Cardillo TM, Furman, RR, Chen, S, Chang, C-H, Goldenberg, DM. Therapy of B-cell malignancies by anti-HLA-DR humanized monoclonal antibody, IMMU-114, is mediated through hyper-activation of ERK and JNK MAP kinase signaling pathways. Submitted for publication.

REFERENCES

1. Molina A. A decade of rituximab: improving survival outcomes in non-Hodgkin's lymphoma. Annu Rev Med 2008;59:237-50.
2. Dimopoulos MA, Gertz MA, Kastritis E, et al. Update on treatment recommendations from the Fourth International Workshop on Waldenstrom's Macroglobulinemia. J Clin Oncol 2009;27:120-6.
3. Mueller BU, Bennett CM, Feldman HA, et al. One year follow-up of children and adolescents with chronic immune thrombocytopenic purpura (ITP) treated with rituximab. Pediatr Blood Cancer 2009;52:259-62.
4. Dalakas MC. B cells as therapeutic targets in autoimmune neurological disorders. Nat Clin Pract Neurol 2008;4:557-67.
5. Zarkhin V, Li L, Kambham N, Sigdel T, Salvatierra O, Sarwal MM. A randomized, prospective trial of rituximab for acute rejection in pediatric renal transplantation. Am J Transplant 2008;8:2607-17.
6. Furst DE, Keystone EC, Kirkham B, et al. Updated consensus statement on biological agents for the treatment of rheumatic diseases, 2008. Ann Rheum Dis 2008;67:Suppl 3:iii2-25.
7. Leonard JP, Martin P, Barrientos J, Elstrom R. Targeted treatment and new agents in diffuse large B-cell lymphoma. Semin Hematol 2008;45:S11-6.
8. Ramos-Casals M, Brito-Zeron P, Munoz S, Soto MJ. A systematic review of the off-label use of biological therapies in systemic autoimmune diseases. Medicine (Baltimore) 2008;87:345-64.
9. Roccatello D, Baldovino S, Alpa M, et al. Effects of anti-CD20 monoclonal antibody as a rescue treatment for ANCA-associated idiopathic systemic vasculitis with or without overt renal involvement. Clin Exp Rheumatol 2008;26:S67-71.

10. Venkateshan SP, Sidhu S, Malhotra S, Pandhi P. Efficacy of biologicals in the treatment of rheumatoid arthritis: a meta-analysis. Pharmacology 2009;83:1-9.
11. McLaughlin P, Grillo-Lopez AJ, Link BK, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four dose treatment program. J Clin Oncol 1998;16:2825-33.
12. Friedberg JW, Neuberg D, Gribben JG, et al. Combination immunotherapy with rituximab and interleukin 2 in patients with relapsed or refractory follicular non-Hodgkin's lymphoma. Br J Haematol 2002;117:828-34.
13. Gisselbrecht C. Use of rituximab in diffuse large B-cell lymphoma in the salvage setting. Br J Haematol 2008;143:607-21.
14. Mounier N, Briere J, Gisselbrecht C, et al. Rituximab plus CHOP (R-CHOP) overcomes bcl-2--associated resistance to chemotherapy in elderly patients with diffuse large B-cell lymphoma (DLBCL). Blood 2003;101:4279-84.
15. Robak T. Novel monoclonal antibodies for the treatment of chronic lymphocytic leukemia. Curr Cancer Drug Targets 2008;8:156-71.
16. Dewhirst MW, Thrall DE, MacEwen EG Spontaneous Pet Animal Cancers. *In:* B. A. Teicher (ed.), Tumor Models in Cancer Research., pp. 565-89. Totowa, NJ: Humana Press, 2001.
17. Hansen K, Khanna C. Spontaneous and genetically engineered animal models: use in preclinical cancer drug development. Eur J Can 2004;40:858-80.
18. Paoloni M, Khanna C. Translation of new cancer treatments from pet dogs to humans. Nature Reviews Cancer 2008;8:147-56.
19. Vail DM, Young KM Canine lymphoma and lymphoid leukemia. *In:* S. J. Withrow and D. M. Vail (eds.), Small Animal Clinical Oncology., 4 edition, pp. 699-733. St. Louis (MO): Saunders, 2007.

20. Modiano JF, Breen M, Burnett RC, et al. Distinct B-cell and T-cell lymphoproliferative disease prevalence among dog breeds indicates heritable risk. Cancer Res 2005;65:5654-61.

21. Breen M, Modiano JF. Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans - man and his best friend share more than companionship. Chromosome Res 2008;16:145-54.

22. Brown KS, Levitt DJ, Shannon M, Link BK. Phase II trial of Remitogen (humanized 1D10) monoclonal antibody targeting class II in patients with relapsed low-grade or follicular lymphoma. Clin Lymphoma 2001;2:188-90.

23. DeNardo GL, Tobin E, Chan K, Bradt BM, DeNardo SJ. Direct antilymphoma effects on human lymphoma cells of monotherapy and combination therapy with CD20 and HLA-DR antibodies and ^{90}Y-labeled HLA-DR antibodies. Clin Cancer Res 2005;11:7075s-9s.

24. Stein R, Qu Z, Chen S, Solis D, Hansen HJ, Goldenberg DM. Characterization of a humanized IgG4 anti-HLA-DR monoclonal antibody that lacks effector cell functions but retains direct antilymphoma activity and increases the potency of rituximab. Blood 2006;108:2736-44.

25. Dechant M, Bruenke J, Valerius T. HLA class II antibodies in the treatment of hematologic malignancies. Semin Oncol 2003;30:465-75.

26. Shi JD, Bullock C, Hall WC, et al. In vivo pharmacodynamic effects of Hu1D10 (remitogen), a humanized antibody reactive against a polymorphic determinant of HLA-DR expressed on B cells. Leuk Lymphoma 2002;43:1303-12.

27. Ravetch J, Kinet J-P. Fc receptors. Annu Rev Immunol 1991;9:457-92.

28. Stein R, Qu Z, Cardillo TM, et al. Antiproliferative activity of a humanized anti-CD74 monoclonal antibody, hLL1, on B-cell malignancies. Blood 2004;104:3705-11.

29. Stein R, Qu Z, Chen S, et al. Characterization of a new humanized anti-CD20 monoclonal antibody, IMMU-106, and its use in combination with the humanized anti-

CD22 antibody, epratuzumab, for the therapy of non-Hodgkin's lymphoma. Clin Cancer Res 2004;10:2868-78.

30. Leung SO, Goldenberg DM, Dion AS, et al. Construction and characterization of a humanized, internalizing, B-cell (CD22)-specific, leukemia/lymphoma antibody, LL2. Molecular Immunol 1995;32:1416-27.

31. Sharkey RM, Juweid M, Shevitz J, et al. Evaluation of a complementarity-determining region-grafted (humanized) anti-carcinoembryonic antigen monoclonal antibody in preclinical and clinical studies. Cancer Res 1995;55:5935s-45s.

32. Cardarelli PM, Quinn M, Buckman D, et al. Binding to CD20 by anti-B1 antibody or $F(ab')_2$ is sufficient for induction of apoptosis in B-cell lines. Cancer Immunol Immunother. 2002;51:15-24.

33. Veterinary co-operative oncology group – common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.0. Vet Comparative Oncol 2004;2:194-213.

34. Nagy ZA, Mooney NA. A novel, alternative pathway of apoptosis triggered through class II major histocompatibility complex molecules. J Mol Med 2003;81:757-65.

35. Mone AP, Huang P, Pelicano H, et al. Hu1D10 induces apoptosis concurrent with activation of the AKT survival pathway in human chronic lymphocytic leukemia cells. Blood 2004;103:1846-54.

36. Newell MK, VanderWal IJ, Beard KS, Freed JH. Ligation of major histocompatibility complex class II molecules mediates apoptotic cell death in resting B lymphocytes. . Proc Natl Acad Sci U S A 1993;90:10459-63.

37. Truman JP, Choqueux C, Tschopp J, et al. HLA class II-mediated death is induced via Fas/Fas ligand interactions in human splenic B lymphocytes. Blood 1997;89:1996-2007.

38. Altomonte M, Visintin A, Tecce R, et al. Targeting of HLA-DR molecules transduces agonistic functional signals in cutaneous melanoma. J Cell Physiol 2004;200:272-6.

39. Aoudjit F, Guo W, Gagnon-Houde JV, et al. HLA-DR signaling inhibits Fas-mediated apoptosis in A375 melanoma cells. Exp Cell Res 2004;299:79-90.

40. Fernández EM, O'Toole PJ, Morrison IE, Cherry RJ, Fernández N. Interaction of HLA-DR with actin microfilaments. Hum Immunol 2003;64:327-37.

41. Guo W, Mourad W, Charron D, Al-Daccak R. Ligation of MHC class II molecules differentially upregulates TNF beta gene expression in B cell lines of different MHC class II haplotypes. Hum Immunol 1999;60:312-22.

42. Coral S, Pucillo C, Leonardi A, Fonsatti E, Altomonte M, Maio M. Triggering of HLA-DR antigens differentially modulates tumor necrosis factor alpha release by B cells at distinct stage of maturation. Cell Growth Differ 1997;8:581-8.

43. Mourad W, Geha RS, Chatila T. Engagement of major histocompatibility complex class II molecules induces sustained, lymphocyte function-associated molecule 1-dependent cell adhesion. J Exp Med 1990;172:1513-6.

44. Blancheteau V, Charron D, Mooney N. HLA class II signals sensitize B lymphocytes to apoptosis via Fas/CD95 by increasing FADD recruitment to activated Fas and activation of caspases. Human Immunol 2002;63:375-83.

45. Elasser D, Valerius T, Repp R, et al. HLA class II as potential target antigen on malignant B cells for therapy with bispecific antibodies in combination with granulocyte colony-stimulating factor. Blood 1996;87:3803-12.

46. Kabelitz D, Janssen O. Growth inhibition of Epstein-Barr virus transformed B cells by anti-HLA-DR antibody L243: possible relationship to L243-induced down regulation of CD23 antigen expression. Cell Immunol 1989;120:21-30.

Table 1. Characteristic phenotype of a canine lymphoma aspirate (150836).

Murine Abs	% Positive	Mean FL	Humanized mAbs	% Positive	Mean FL
none	3.9	3.4	none	4.5	3.2
Ag8	2.8	3.0	hMN-14	4.6	3.2
L243	77.8	10.4	IMMU-114	26.2	5.5
2B8 (anti-CD20)	2.6	3.1	hA20 (anti-CD20)	4.0	3.3
LL1 (anti-CD74)	6.7	4.0	hLL1 (anti-CD74)	4.7	3.3
LL2 (anti-CD22)	5.1	3.7	hLL2 (anti-CD22)	4.9	3.4

Figure legends

Fig. 1. *In vitro* effects of murine L243 on canine lymphoma aspirates. *A.* L234 binding to the aspirates from 4 dogs. White bars, Ag8; gray bars, L243. ***B.*** Percent apoptotic cells as measured by flow cytometry of hypodiploid DNA (sub G0) following propidium iodine staining. Incubations were performed without second antibody or in the presence of goat anti-mouse IgG. ***C.*** Viable cell count was performed on two of the specimens by flow cytometry analysis of the cell count within a viable gate defined in the forward scatter *vs.* side scatter dot plot. For panels B and C: white bars, Ag8 without second antibody; striped bars, Ag8 with GAM; gray bars, L243 without second antibody; black bars, L243 with GAM; *, $P<0.05$ vs. Ag8.

Fig. 2. *In vitro* effects of IMMU-114 on canine lymphoma aspirates. *A.* Percent apoptotic cells as measured by flow cytometry of hypodiploid DNA (sub G0) following propidium iodine staining. Incubations were performed without second antibody or in the presence of goat anti-mouse IgG (GAM) or goat anti-human IgG (GAH). ***B* and *C.*** Percent specific lysis in CDC and ADCC assays on aspirate of dog #171205. Error bars, SD of three replicates. *, significant change ($P<0.05$) relative to no mAb control.

Fig 3. Peripheral blood lymphocyte count and lymphocyte subset phenotyping indicated a decrease in both B- and T-cell lymphocytes. ■, total lymphocyte count; ●, T-cell count, relative to baseline; ○, B-cell count, relative to baseline.

Fig. 4. Flow cytometry overlays showing the targeting of L243 to dog lymphoma cells *in vivo*. A comparison of baseline and lymph node aspirate cells removed one week after first L243 infusion. Panel A, untreated cells, no first or second antibodies were added in vitro. Panel B, cells were incubated in vitro only with FITC labeled GAM. Panel C, cells were incubated in vitro with L243 and FITC-GAM. Panel D, cells were incubated in vitro with Ag8 and FITC-GAM

Mean fluorescence values of histogram peaks are shown as insets in each panel. Gray line, baseline; black filled histogram, day-7 post-treatment aspirate.

Fig. 5. Clearance of L243 in a dog with lymphoma (A) and of IMMU-114 in two normal dogs (B and C). The L243 doses administered in the dog with lymphoma (A) were 1.5 mg/kg for treatment 1 and 3.0 mg/kg for the remaining 3 treatments. IMMU-114 doses were 3.0 mg/kg for the initial dose in both normal dogs (B and C). The second dose of IMMU-114 administered to second normal dog (C) was 1.3 mg/kg.